

Research article

The inhibitory activity of ginsenoside Rp4 in adenosine diphosphate-induced platelet aggregation



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ARTICLE INFO

Article history:

Received 4 December 2015

Received in Revised form

15 January 2016

Accepted 26 January 2016

Available online 15 February 2016

Keywords:

fibrinogen

ginsenoside Rp4

granule

MAPK

platelet aggregation

ABSTRACT

Background: Korean ginseng, *Panax ginseng* Meyer, has been used as a traditional oriental medicine to treat illness and promote health for several thousand years. Ginsenosides are the main constituents for the pharmacological effects of *P. ginseng*. Since several ginsenosides, including ginsenoside (G)-Rg3 and G-Rp1, have reported antiplatelet activity, here we investigate the ability of G-Rp4 to modulate adenosine diphosphate (ADP)-induced platelet aggregation. The ginsenoside Rp4, a similar chemical structure of G-Rp1, was prepared from G-Rg1 by chemical modification.

Methods: To examine the effects of G-Rp4 on platelet activation, we performed several experiments, including antiplatelet ability, the modulation of intracellular calcium concentration, and P-selectin expression. In addition, we examined the activation of integrin $\alpha\text{IIb}\beta_3$ and the phosphorylation of signaling molecules using fibrinogen binding assay and immunoblotting in rat washed platelets.

Results: G-Rp4 inhibited ADP-induced platelet aggregation in a dose-dependent manner. We found that G-Rp4 decreased calcium mobilization and P-selectin expression in ADP-activated platelets. Moreover, fibrinogen binding to integrin $\alpha\text{IIb}\beta_3$ by ADP was attenuated in G-Rp4-treated platelets. G-Rp4 significantly attenuated phosphorylation of extracellular signal-regulated protein kinases 1 and 2, p38, and c-Jun N-terminal kinase, as well as protein kinase B, phosphatidylinositol 3-kinase, and phospholipase C- γ phosphorylations.

Conclusion: G-Rp4 significantly inhibited ADP-induced platelet aggregation and this is mediated via modulating the intracellular signaling molecules. These results indicate that G-Rp4 could be a potential candidate as a therapeutic agent against platelet-related cardiovascular diseases.

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1. Introduction

Cardiovascular diseases (CVDs) are the highest cause of death in Western and Asian countries due to epidemiological transition, lifestyle changes, nutrition transition, and economic consequences in the modern world [1,2]. Recent studies have suggested that CVDs are mainly caused by abnormal platelet activation and aggregation. Atherosclerosis, one of the major CVDs, is a chronic inflammatory disease in the arterial vessel wall, and thus, causing plaque and thrombus formation [3,4].

Platelets, derived from megakaryocytes of bone marrow, are important factors relating to the progression of atherosclerosis.

Platelets play an essential role in hemostasis, thrombosis, inflammatory processes, atherosclerosis, and tumor metastasis [5–7]. When there is vascular injury, platelets exposed to subendothelial matrix components including von Willebrand factor and collagen trigger adhesion and activation [8]. Platelet activation leads to the secretion of platelet granules including insulin-like growth factor 1, platelet-derived growth factor, transforming growth factor-beta, platelet factor 4, adenosine diphosphate (ADP), adenosine triphosphate, and serotonin, which contain other platelet agonists such as ADP, collagen, thrombin, and the generation of lipid-based agonists such as thromboxane A₂, resulting in an increase in intracellular calcium ion ($[\text{Ca}^{2+}]_i$) and platelet shape change. These

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secondary agonists amplify the signals leading to fully platelet-cascade activation [9].

ADP plays an essential role in the process of hemostasis by signaling through G-protein coupled receptors (GPCR), P2Y1 and P2Y12 receptors. The Gq-coupled P2Y1 receptor plays a role in ADP-induced platelet shape change. P2Y1 receptor signaling is important for GPCR-mediated integrin activation, platelet granule release, and consequently platelet aggregation [10]. In addition, Gi-coupled-P2Y12 receptor induces platelet activation in the presence of the GPCR agonist ADP. The role of P2Y12 receptors in promoting platelet aggregation is consistent with inhibitory effects on cAMP, which relieves the inhibitory effect of cAMP-dependent protein kinase on platelet activation.

Korean ginseng (*Panax ginseng* Meyer) has been used as a traditional Korean medicine. This folk medicine has been used to treat illness and promote health for several thousand years [11]. The therapeutic potential of Korean ginseng has been discussed in a multitude of *in vitro* and *in vivo* studies [12]. Recently ginseng's marvelous effects have been studied in a wide range of pathological conditions, such as CVDs, immune diseases, ischemic heart disease, stroke, nervous disorders, and cancer [12]. Moreover, the ginseng-mediated protective mechanism is reported to be due to antioxidant, anti-inflammatory, antiaging, and antiapoptotic activity. In addition, the antiplatelet activities by ginseng extracts and some ginsenosides have also been determined [13,14]. However, information on the effect of ginsenoside (G)-Rp4 on platelet activation is limited. G-Rp4, a derivative from G-Rg1, is verified to be chemically stable in comparison to G-Rg1.

In this study, the antiplatelet properties of G-Rp4 were investigated using ADP-stimulated platelet aggregation. We further determined whether G-Rp1 affected intracellular downstream signal transductions (i.e., intracellular calcium ion concentration, granule secretions, signaling molecule phosphorylation, and fibrinogen binding to active integrin $\alpha_{IIb}\beta_3$).

2. Materials and methods

2.1. Materials

G-Rp4 with 99% purity was obtained from Ambo Institute (Seoul, Korea). ADP was purchased from Chronolog (Havertown, PA, USA). Fura-2-acetoxymethyl ester (Fura-2/AM) was procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse monoclonal to CD62P antibody and goat polyclonal to mouse immunoglobulin (Ig) G-H&L (fluorescein isothiocyanate) antibodies were gained from Abcam (Cambridge, UK). Alexa Fluor488 fibrinogen conjugate was from Molecular Probes (Eugene, OR, USA). All other chemicals were of reagent grade. Antibodies of phospho-extracellular signal-regulated kinase-2 (ERK2; p44/42), total-ERK2 (p44/42), phospho-p38, total-p38, phospho-c-Jun N-terminal kinase (JNK), total JNK, phospho-protein kinase B (Akt), total Akt, phospho-phosphatidylinositol 3-kinase (PI3K; p85/p55), and PI3K (p85), phospho-phospholipase C (PLC)- γ , total PLC γ , were purchased from Cell Signaling (Beverly, MA, USA).

2.2. Washed rat platelet preparation

Male Sprague Dawley 8–10-wk old rats (240–250g) were acquired from Orient Co. (Seoul, Republic of Korea). The rats were maintained in a standard laboratory animal facility with free access to food and water and they were acclimatized to these conditions for at least 1 wk before use. Whole blood from the rats was collected using a 23G needle inserted into the left ventricle of the heart and then transferred to a 14-mL round test tube including 1 mL of acid citrate dextrose solution (85mM trisodium citrate,

83mM dextrose, and 21mM citric acid) as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of the whole blood samples at 350 g for 7 min twice at room temperature, and the PRP was washed with washing buffer (Tyrode buffer 4). Washed platelets were then tenderly resuspended in Tyrode buffer (137mM NaCl, 12mM NaHCO₃, 5.5mM glucose, 2mM KCl, 1mM MgCl₂, 0.3mM NaHPO₄, pH 7.4) to a final concentration of 3×10^8 platelets/mL [15].

2.3. Platelet aggregation *in vitro* assay

Platelet aggregation was performed as previously described [16,17]. PRP (3×10^8 platelets/mL) was preincubated for 2 min at 37°C in the presence of 1mM exogenous CaCl₂ with various concentrations (6.25–50 μ M) of G-Rp4 and then platelet aggregation was encouraged by ADP (10 μ M). The reaction mixture was further incubated for 5 min with stirring at 250 g. The concentration of the vehicle was held at < 0.05%. The aggregation was monitored using an aggregometer (Chronolog, Havertown, PA, USA) at a constant stirring of 250 g and aggregation rates were measured as the light transmission changes were recorded for 9 min.

2.4. [Ca²⁺]_i measurement

The [Ca²⁺]_i was measured with Fura-2/AM as previously described [15]. Briefly, the washed platelets were incubated with Fura-2/AM (5 μ M) for 30 min at 37°C and platelet aggregation assay was done using the washed platelets. The Fura-2-loaded platelets (3×10^8 /mL) were then preincubated with G-Rp4 for 2 min at 37°C in the presence of 1mM CaCl₂, and subsequently stimulated with ADP for 5 min. Fluorescent signals were recorded using a F-2500 fluorescence spectrofluorometer (Hitachi, Ltd., Tokyo, Japan). Light emission was measured at 510 nm with simultaneous excitation at 340 nm and 380 nm that changed every 0.5 s. Fura-2 fluorescence in the cytosol measured with the spectrofluorometer was calculated as previously described [18,19].

2.5. Determination of P-selectin expression

Washed platelets, treated with various concentrations (6.25–50 μ M) of G-Rp4 or vehicle were stimulated with ADP (10 μ M) and incubated at 37°C for 5 min. The reaction was discontinued, and platelets were centrifuged consecutively and resuspended in cool phosphate-buffered saline (PBS), containing 10% fetal bovine serum and 1% sodium azide. Platelets were then incubated with CD62P primary antibody in 3% bovine serum albumin/PBS for 30 min at 4°C in the dark. Next, platelets were fixed using 0.5% paraformaldehyde and washed three times by centrifugation at 2,000 \times g for 5 min and resuspended in cool PBS one after another. Fluorescein isothiocyanate-conjugated secondary antibody was incubated in 3% bovine serum albumin/PBS for 30 min at 4°C in the dark. Flow cytometry analysis was performed on a FACSAria III flow cytometer using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) [11].

2.6. Determination of fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

Washed platelets were at first treated with G-Rp4 or vehicle and incubated for 5 min at room temperature. Then, 10 μ M ADP was added together with 200 μ g/mL Alexa Fluor 488-human fibrinogen (Molecular Probes, Eugene, OR, USA), and the sample was incubated at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACSAria III flow cytometer (Becton Dickinson Immunocytometry Systems), and

data were analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems) [11].

2.7. Immunoblotting

Platelets (5×10^8 /mL) were activated with ADP for 5 min in the presence of 1mM CaCl_2 with G-Rp4 (6.25–50 μM) and instantly dissolved in sample buffer (0.125M Tris-HCl at pH 6.8, 2% fetal bovine serum, 2% β -mercaptoethanol, 20% glycerol, 0.02% bromophenol blue in the presence of 1mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 $\mu\text{g}/\text{mL}$ pepstatin). Protein concentration was measured using bicinchoninic acid assay (PRO-MEASURE, iNtRON Biotechnology, Kyungki-Do, Korea) on ice. After boiling for 5 min, the proteins were resolved by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes in a transfer buffer [25mM Tris (pH 8.5) and 20% methanol]. The membrane was blocked with 5% skim milk, washed, and subjected to immunoblotting with ERK1/2, p38 JNK, Akt, PI3K, PLC γ antibodies. The immunoblots were again incubated with horseradish peroxidase secondary antibody and the membranes were visualized using enhanced chemiluminescence (iNtRON Biotechnology).

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) tool, followed by a *post hoc* Dunnett's test in order to determine the statistical significance of the differences between treatment groups. All data are presented as means \pm standard deviation, and $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of G-Rp4 on ADP-induced platelet aggregation

The concentration of ADP (10 μM) could induce full activation and aggregation in rat washed platelets. Therefore, 10 μM of ADP was used as the ligand to induce platelet aggregation in the further studies. While washed platelets were strongly activated by 10 μM of ADP in the presence of 1mM CaCl_2 , G-Rp4 significantly reduced ADP-induced platelet aggregation in a concentration-dependent manner with an IC_{50} of $30.86 \pm 0.12\mu\text{M}$ (Fig. 1B). However, G-Rg1

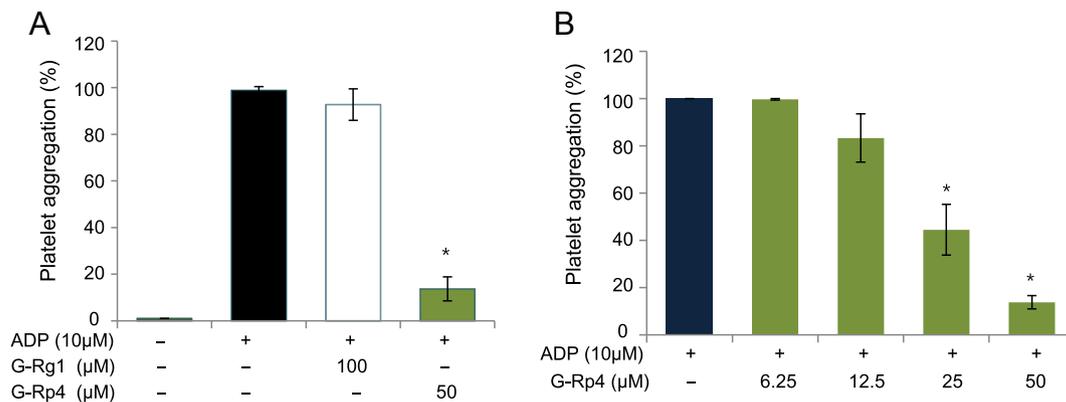


Fig. 1. The inhibitory effect of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-induced platelet aggregation. (A) Platelets (3×10^8 /mL) were preincubated with or without G-Rg1 (100 μM) and G-Rp4 (50 μM) in the presence of 1mM CaCl_2 at 37°C for 2 min. (B) Platelets (3×10^8 /mL) were preincubated with or without G-Rp4 (6.25–50 μM) in the presence of 1mM CaCl_2 at 37°C for 2 min. The platelet aggregation was induced by 10 μM of ADP, and the extent of aggregation was determined with chrono-log aggregometer. The aggregation reaction was completed after 5 min, and the percent aggregation rate was calculated. The bar graphs are a summary of four independent experiments. Each column shows the mean \pm standard deviation of at least three independent experiments. * $p < 0.001$ versus ADP-activated control.

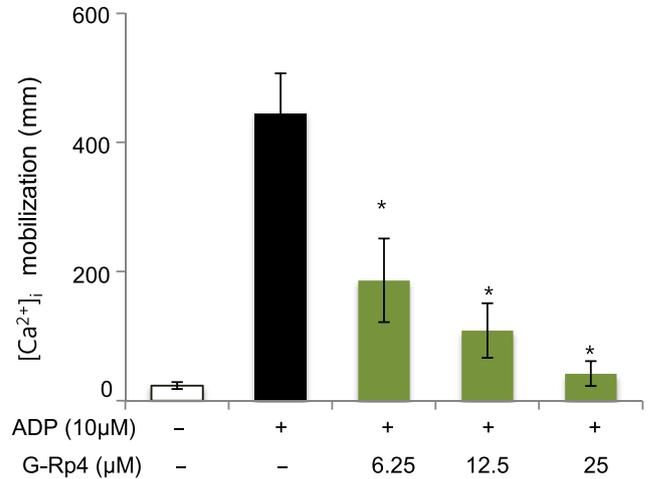


Fig. 2. Effect of ginsenoside (G)-Rp4 in adenosine diphosphate (ADP)-activated [Ca^{2+}]_i concentration. Washed platelets (3×10^8 /mL) were incubated with or without a G-Rp4 in the presence of 1mM CaCl_2 at 37°C for 2 min. The platelets were stimulated with ADP (10 μM) at 37°C for 3 min. Intracellular calcium ion was then determined with a spectrofluorometer as described in the Materials and methods section. * $p < 0.001$ versus ADP-stimulated control. [Ca^{2+}]_i, intracellular calcium ion.

has not shown any antiplatelet activity in ligand-induced platelet aggregation (Fig. 1A).

3.2. G-Rp4 reduces ADP-induced [Ca^{2+}]_i mobilization

The release of granule contents boosts platelet activation signals and is considered to be an important indicator of the platelet-activating cascade. [Ca^{2+}]_i concentration plays an essential role in platelet aggregation such as platelet activation and thrombus formation. Thus, we investigated the effects of G-Rp4 on ADP-induced [Ca^{2+}]_i mobilization. Pretreatment of the washed platelets with the various concentration of G-Rp4 (6.25–25 μM) remarkably prevented the rise in ADP-stimulated [Ca^{2+}]_i (Fig. 2).

3.3. G-Rp4 inhibits P-selectin expression in ADP-activated platelets

Since granule secretions are important markers of platelet activation in the earlier phase of platelet aggregation, we determined whether G-Rp4 preincubation affected the granule release in

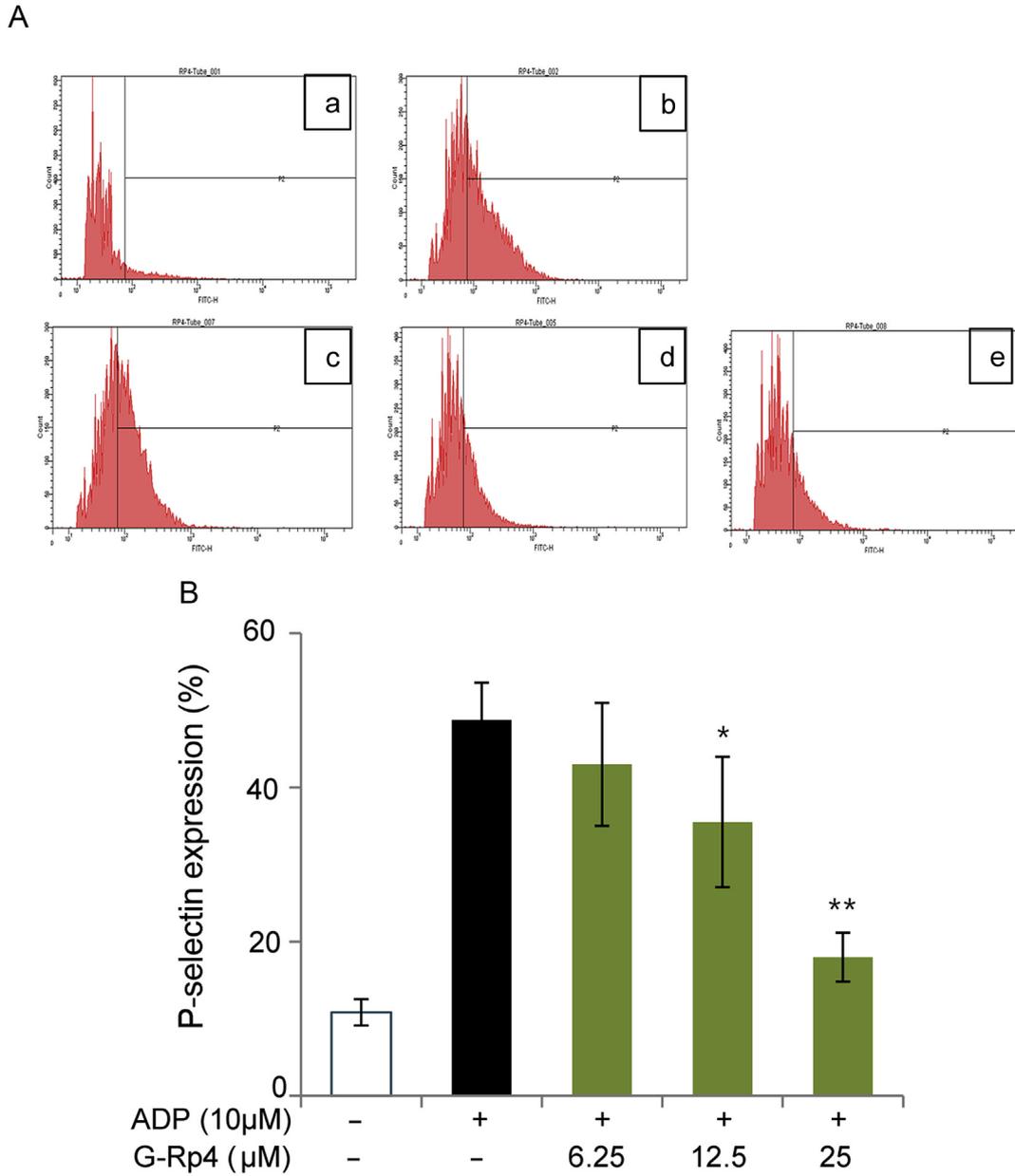


Fig. 3. Effect of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-activated P-selectin expression. Washed platelets (3×10^8 /mL) were incubated with or without a G-Rp4 (6.25–25 μ M) in the presence of 1mM CaCl₂ at 37°C for 2 min. The platelets were stimulated with adenosine diphosphate (10 μ M) for 3 min at 37°C. (A) Flow cytometry analysis was performed on a FACSAria III flow cytometer using CellQuest software. (B) The data are presented as mean \pm standard deviation of three independent experiments. * $p < 0.01$. ** $p < 0.001$ versus ADP-activated control. FITC, fluorescein isothiocyanate.

ADP-stimulated platelets. ADP-stimulated P-selectin secretion was remarkable and concentration-dependently reduced in G-Rp4 treated platelets (Figs. 3A and 3B). It showed that platelet α -granule secretory activity was significantly and dose-dependently impaired by G-Rp4 pretreatment.

3.4. G-Rp4 reduces fibrinogen binding to activated integrin- α IIb β 3

The ligation of integrin- α IIb β 3 function is the principal consequence of activation and adhesion in platelets and then aggregation as a result of the adhesive substrates bound to the membranes of activated platelets. Therefore, we determined the function of G-Rp4 on the response mechanism in integrin α IIb β 3 activation. ADP-stimulated fibrinogen binding to its receptor was concentration-dependently inhibited in various G-Rp4-treated platelets

(Fig. 4). As expected, ethylene glycol tetra-acetic acid (10 μ M) significantly blocked fibrinogen binding to active integrin- α IIb β 3 [20]. This result suggested that G-Rp4 may impair integrin- α IIb β 3 conformational changes for high correspondence fibrinogen binding site exposure (inside-out signaling) that arises as a result of prior platelet agonist interactions [21–23]. The next step of outside-in signaling implies a number of intracellular effectors, which is essentially associated with the integrin β 3 subunit, PLC γ and PI3K [24–26].

3.5. G-Rp4 suppresses ADP-stimulated platelet mitogen-activated protein kinases phosphorylations

The antiplatelet effect is known to inhibit phosphorylation of mitogen-activated protein kinases (MAPKs; p38 MAPK, ERK, and

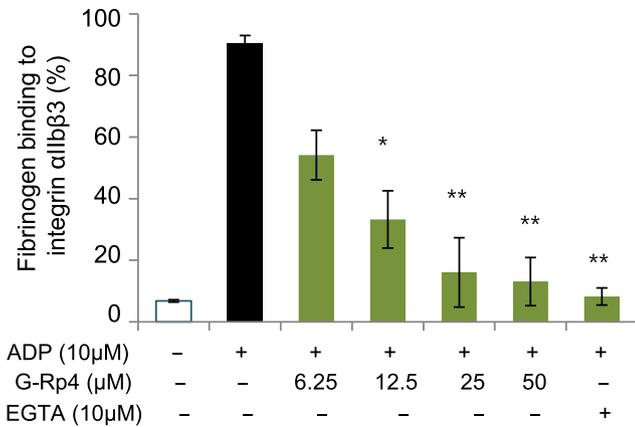


Fig. 4. Ginsenoside (G)-Rp4 reduces fibrinogen binding to activated integrin- α IIb β 3. The inhibitory effect of G-Rp4 in fibrinogen binding to integrin- α IIb β 3 in adenosine diphosphate (ADP)-stimulated platelets was examined using flow cytometry analysis. Washed platelets (3×10^8 /mL) were pretreated CaCl₂ 1mM with or without G-Rp4 at 37°C for 2 min and then the platelets were added to 10μM ADP with Alexa Fluor 488-human fibrinogen (200 μg/mL), and the sample was incubated at 37°C for 15 min. The bar graphs shows the mean \pm standard deviation of three independent experiments. * $p < 0.05$. ** $p < 0.001$ versus ADP-activated control. EGTA, ethylene glycol tetra-acetic acid.

4. Discussion

Ginseng contains saponins, proteins, amino acids, nucleic acids, fatty acid components, essential oils, polyacetylenes, phenolic compounds, terpenoids, and various other components. Among them, ginsenosides are the pharmacologically active components found in ginseng that are classified into 20(S)-protopanaxatriols such as Re and Rg1 and 20(S)-protopanaxadiols like Rg2 and Rh1, according to a site of glycoside [32].

Until now, research on ginseng has been carried out in order to investigate its pharmacological efficacy and various other medical and biological aspects. Ginsenosides are known to exhibit various effects by acting on physical functions such as central nervous, endocrine, immune, and metabolic systems [11,33–35]. Among these ginsenosides, Rg1 was reported to be efficacious for antifatigue and memory progression. Besides, G-Rp1 derived from G-Rg3 was reported to be potent in antiplatelet activity, but information on the effect of the stable compound G-Rp4 derived from G-Rg1 on platelet activation is limited. Therefore, we first examined whether G-Rp4 affected ADP-induced platelet aggregation, and then investigated its molecular mechanism of antiplatelet activity by analyzing downstream signal transduction (e.g., [Ca²⁺]_i mobilization, P-selectin secretion, MAPK and PI3K/Akt phosphorylation, and fibrinogen binding to active integrin α -IIb β 3).

Previous reports showed that G-Rp1 has potent antiplatelet activity through the mechanisms of PI3K, PLC γ , MAPKs, and phosphorylation of vasodilator-stimulated phosphoprotein by increasing cAMP [11]. In the present study, we have shown that G-Rp4 mediates its antiplatelet activity by phosphorylation of PLC γ , PI3K, Akt, and MAPKs through signaling of GPCR P2Y₁₂ binding to ADP.

Platelets, also called thrombocytes, are small, irregular-shaped blood cells derived from megakaryocytes that serve diverse roles in different pathological or physiological pathways in hemostasis and inflammation [13]. Platelet activation and aggregation occurs via many biochemical and biophysical pathways. Platelet agonists, such as collagen, ADP, thrombin, and thromboxane A₂, activate platelets through GPCR pathways [36]. Platelets are usually present in the inactive state, but activation with agonists like collagen, ADP, thrombin, and thromboxane A₂ turn on the active state of platelets [37]. Platelet aggregation occurs when membrane receptors bind to

JNK) and is also reported to be phosphorylated by various platelet agonists [27–30]. Thus, we experimented whether G-Rp4 could modulate MAPKs. Our findings showed that G-Rp4 significantly inhibited ADP-induced ERK1/2, JNK, and p38 MAPKs signaling pathway in a concentration-dependent manner (Figs. 5A and 5B).

3.6. G-Rp4 inhibits the phosphorylation of PLC γ and PI3K/AKT proteins involved in platelet glycoprotein signaling

PI3K plays a remarkable role in platelet-ADP receptor signaling pathway. Platelet glycoproteins and the activation of PLC γ is significant for the most part of functional reactions to ADP, containing aggregation and alpha-granule secretion, so here we demonstrated the effect of G-Rp4 on the expression levels of the indicated proteins [31]. G-Rp4 suppressed ADP-stimulated PLC γ and PI3K/AKT phosphorylation in a dose-dependent manner (Fig. 6).

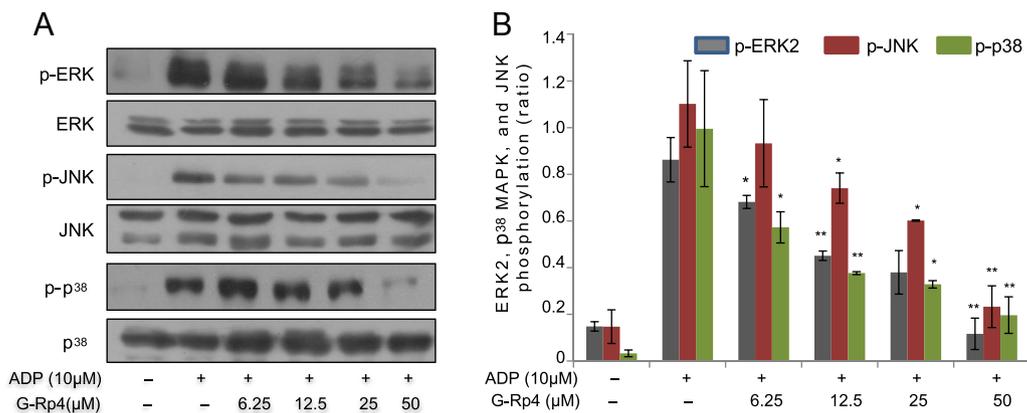


Fig. 5. Effects of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-stimulated phosphorylation of mitogen-activated protein kinases (MAPKs). Washed platelets (3×10^8 /mL) were incubated for 2 min at 37°C with vehicle or G-Rp4 at the indicated concentration in the presence of 1mM CaCl₂ with stirring. The platelets were then stimulated with 10μM ADP for 5 min at 37°C. After completing the responses, total platelet proteins were extracted. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. (A) The membranes were then investigated with antibodies against total and phospho-extracellular-signal-regulated kinases 1 and 2 (ERK1/2), p38, stress-activated protein kinases/Jun amino-terminal kinases (JNK). Antibody binding was visualized with chemiluminescence. All immunoblots are representative of three or four independent experiments. (B) The bar graph shows the mean \pm standard deviation of three or four independent experiments. * $p < 0.05$. ** $p < 0.001$ versus control.

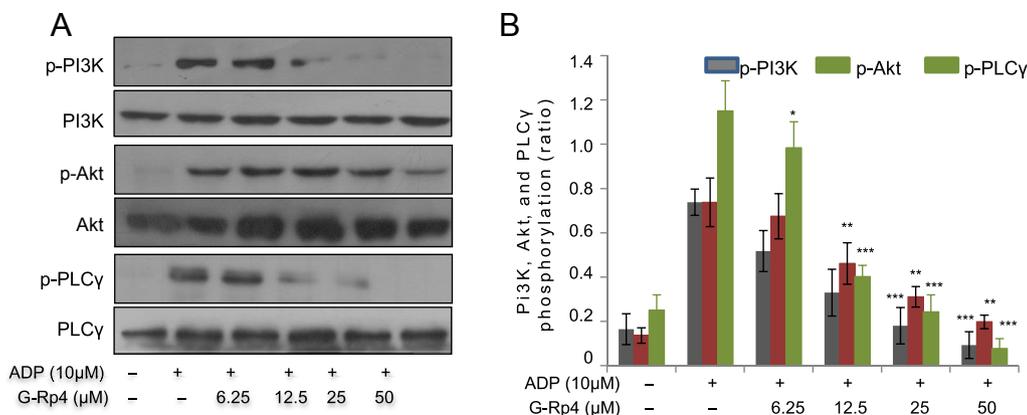


Fig. 6. Effects of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-stimulated phosphorylation of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and phospholipase C- γ (PLC γ). Washed platelets (3×10^8 /mL) were preincubated at 37°C for 2 min with vehicle or various concentration of G-Rp4. The platelets were then stimulated with 10 μ M ADP at 37°C for 5 min. After completing the reactions, total platelet proteins were extracted. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. (A) The membranes were then incubated with antibodies against phospho-PLC γ , phospho-PI3K, and phospho-Akt. Antibody binding was visualized with chemiluminescence. (B) The bar graph shows the mean \pm standard deviation of three or four independent experiments. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$ versus vehicle control.

ligands, or the alkaline concentration of platelet cytosolic increases due to the activation of various signal transduction factors. With the binding of ADP to P2Y1 receptors, Gq protein target and activate PLC which cleavage phosphatidylinositol 4,5-bisphosphate into inositol triphosphate and diacylglycerol. The resulting inositol triphosphate causes an increase in the $[Ca^{2+}]_i$ level in platelets [10]. Subsequently, platelet shape change is caused by contraction of cytoskeletal protein. However, diacylglycerol and calcium work in conjunction causing the increased level of PKC which goes on to phosphorylate other molecules involved in platelet cellular activity. In this cellular cascade, platelet activation facilitates platelet aggregation and granule secretion in the platelets. Here, we confirmed that $[Ca^{2+}]_i$ concentration and P-selectin expression are increased when platelets are treated with ADP, which was inhibited with G-Rp4 treatment. Since granule release is known to occur in the early phase of platelet activation and aggregation [38,39], this indicated that G-Rp4 affected the initial stage of platelet activation.

In this study, we demonstrated that G-Rp4 remarkably inhibited ADP-stimulated MAPK phosphorylation (e.g., ERK1/2, JNK, and p38 MAPK), suggesting that MAPK signaling may be involved in G-Rp4's antiplatelet activity. It was well known that ERK1/2 cascade in platelet activation was involved in the $[Ca^{2+}]_i$ mobilization in ADP-stimulated platelets. Moreover, it is well-established that JNK and p38 MAPK signaling play an important role in stimulating granule secretion and integrin α IIb β 3 activation agonist-induced platelet aggregation and thrombus formation [40,41]. This is the case with PI3K/Akt signaling in agonist-activated platelet aggregation. PI3K/Akt is involved in the ligand-stimulated platelet activation, which was mediated via PI3K/Akt-nitric oxide synthase-cyclic guanosine monophosphate (GMP) axis [42]. Although we have yet to determine the involvement of nitric oxide-cyclic GMP, we inferred to the participation of PI3K/Akt-cyclic GMP pathway in G-Rp4-mediated antiplatelet activity. Interestingly, G-Rp4 markedly inhibited integrin- α IIb β 3 activation in ADP-stimulated platelets (Fig. 3). Platelet integrin- α IIb β 3 could bind to multiple ligands, such as fibrinogen, fibronectin, and von Willebrand factor, which mediates outside-in signaling and is the final step in platelet activation and aggregation [43,44]. The blocking of integrin- α IIb β 3 activation is a fascinating target in clinical antiplatelet pharmacology [45,46]. Therefore, G-Rp4 could be a candidate of antiplatelet therapeutic for inhibiting the outside-in signaling of integrin- α IIb β 3 directly or indirectly, which remains to be clarified (Fig. 7).

In conclusion (Fig. 7), this study indicates that the inhibitory effect of G-Rp4 occurs in a concentration-dependent manner in ADP-induced platelet aggregation. In addition, G-Rp4 markedly suppressed intracellular downstream signal transduction (i.e., granule secretion and the phosphorylation of signaling molecule proteins). That is, G-Rp4 significantly inhibited ADP-induced $[Ca^{2+}]_i$ mobilization and P-selectin expression. Furthermore, we found that G-Rp4 notably inhibited the phosphorylation of all three MAPKs (i.e., ERK1/2, JNK, and p38 MAPK) and PI3K/Akt phosphorylation. Finally, integrin- α IIb β 3 activation, the final step of full platelet activation, was remarkably suppressed by G-Rp4 pretreatment. Based on our present results, we suggest that G-Rp4 showed antiplatelet activity via the modulation of intracellular downstream

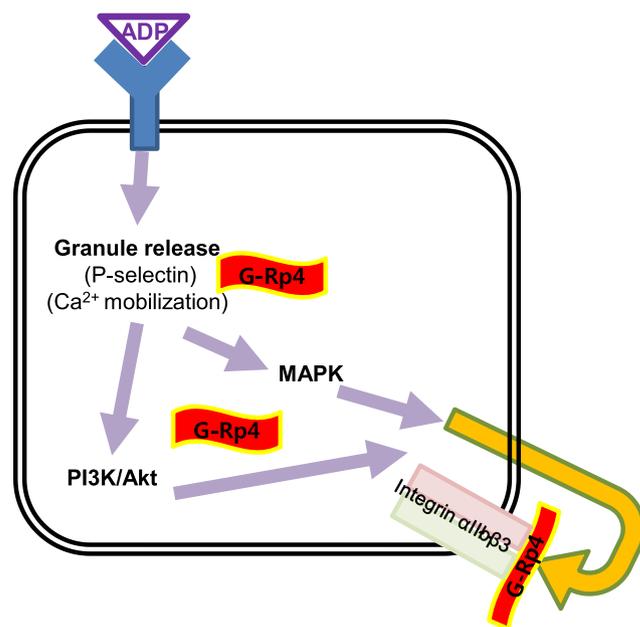


Fig. 7. The schematic diagram for possible inhibitory activity of ginsenoside Rp-4 on adenosine diphosphate (ADP)-induced platelet aggregation. Akt, protein kinase B; G, ginsenoside; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

signaling components. Therefore, G-Rp4 can be regarded as a potential therapeutic agent for preventing CVDs, such as thrombosis and ischemia.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

This research was supported by the National Research Foundation of Korea grant funded by the Korean Government (MSIP; number: 2015R1D1A1A09057204).

References

- Reddy KS, Yusuf S. Emerging epidemic of cardiovascular disease in developing countries. *Circulation* 1998;97:596–601.
- McKeigue PM, Shah B, Marmot MG. Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. *Lancet* 1991;337:382–6.
- Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med* 2007;357:2482–94.
- Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med* 2008;359:938–49.
- Adam F, Kauskot A, Rosa JP, Bryckaert M. Mitogen-activated protein kinases in hemostasis and thrombosis. *J Thromb Haemost* 2008;6:2007–16.
- Dovizio M, Alberti S, Guillem-Llobat P, Patrignani P. Role of platelets in inflammation and cancer: novel therapeutic strategies. *Basic Clin Pharmacol Toxicol* 2014;114:118–27.
- Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest* 2005;115:3378–84.
- Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61–7.
- Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002;8:1227–34.
- Moheimani F, Jackson DE. P2Y12 receptor: platelet thrombus formation and medical interventions. *Int J Hematol* 2012;96:572–87.
- Endale M, Lee WM, Kamruzzaman SM, Kim SD, Park JY, Park MH, Park TY, Park HJ, Cho JY, Rhee MH. Ginsenoside-Rp1 inhibits platelet activation and thrombus formation via impaired glycoprotein VI signalling pathway, tyrosine phosphorylation and MAPK activation. *Br J Pharmacol* 2012;167:109–27.
- Attele AS, Wu JA, Yuan C-S. Ginseng pharmacology: Multiple constituents and multiple actions. *Biochemical Pharmacology* 1999;58:1685–93.
- Jung KY, Kim DS, Oh SR, Lee IS, Lee JJ, Park JD, Kim SI, Lee HK. Platelet activating factor antagonist activity of ginsenosides. *Biol Pharm Bull* 1998;21:79–80.
- Zhang W, Chen G, Deng CQ. Effects and mechanisms of total *Panax notoginseng* saponins on proliferation of vascular smooth muscle cells with plasma pharmacology method. *J Pharm Pharmacol* 2012;64:139–45.
- Lee HS, Kim SD, Lee WM, Endale M, Kamruzzaman SM, Oh WJ, Cho JY, Kim SK, Cho HJ, Park HJ, et al. A noble function of BAY 11-7082: Inhibition of platelet aggregation mediated by an elevated cAMP-induced VASP, and decreased ERK2/JNK1 phosphorylations. *Eur J Pharmacol* 2010;627:85–91.
- Lee DH, Cho HJ, Kang HY, Rhee MH, Park HJ. Total saponin from Korean red ginseng inhibits thromboxane A2 production associated microsomal enzyme activity in platelets. *J Ginseng Res* 2012;36:40–6.
- Park JY, Oh WJ, Kim MJ, Kim TH, Cho JY, Park HJ, Lee IK, Kim S, Kim GS, Kim SK, et al. Mechanism of anti-platelet activity of *Oligoporus tephroleucus* oligoporus A: involvement of extracellular signal-regulated kinase phosphorylation and cyclic nucleotide elevation. *Platelets* 2012;23:376–85.
- Park JY, Ji HD, Jeon BR, Im EJ, Son YM, Lee JY, Lee DH, Lee YC, Hyun E, Jia Q, et al. Chlorin e6 prevents ADP-induced platelet aggregation by decreasing PI3K-Akt phosphorylation and promoting cAMP production. *Evid Based Complement Alternat Med* 2013;2013:569160.
- Schaeffer J, Blaustein MP. Platelet free calcium concentrations measured with fura-2 are influenced by the transmembrane sodium gradient. *Cell Calcium* 1989;10:101–13.
- Phillips DR, Baughan AK. Fibrinogen binding to human platelet plasma membranes. Identification of two steps requiring divalent cations. *J Biol Chem* 1983;258:10240–6.
- Obergfell AEK, Mocsai A, Buensuceso C, Moores SL, Brugge JS, Lowell CA, Shattil SJ. Coordinate interactions of Csk, Src, and Syk kinases with allb3 initiate integrin signaling to the cytoskeleton. *J Cell Biol* 2002;265–75.
- Wonerow PPA, Vaux DJ, Watson SP. A critical role for phospholipase C2 in allb3-mediated platelet spreading. *J Biol Chem* 2003;375:20–9.
- Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A* 2003;100:13298–302.
- Calderwood DA. Integrin activation. *J Cell Sci* 2004;117:657–66.
- Canobbio ISL, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Torti M. Genetic evidence for a predominant role of PI3K catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood* 2009;2193–6.
- Suzuki-Inoue KHC, Inoue O, Kaneko M, Cuyun-Lira O, Takafuta T, Watson SP, Ozaki Y. Involvement of Src kinases and PLCγ2 in clot retraction. *Thromb Res* 2007;120:251–8.
- Woulfe DS. Akt signaling in platelets and thrombosis. *Expert Rev Hematol* 2010;3:81–91.
- Zahid MS, Waise TM, Kamruzzaman M, Ghosh AN, Nair GB, Mekalanos JJ, Faruque SM. The cyclic AMP (cAMP)-cAMP receptor protein signaling system mediates resistance of *Vibrio cholerae* O1 strains to multiple environmental bacteriophages. *Appl Environ Microbiol* 2010;76:4233–40.
- Jackson EC, McNicol A. Cyclic nucleotides inhibit MAP kinase activity in low-dose collagen-stimulated platelets. *Thromb Res* 2010;125:147–51.
- Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost* 2012;10:167–76.
- Mangin P, Nonne C, Eckly A, Ohlmann P, Freund M, Nieswandt B, Cazenave JP, Gachet C, Lanza F. A PLC gamma 2-independent platelet collagen aggregation requiring functional association of GPVI and integrin alpha2beta1. *FEBS Lett* 2003;542:53–9.
- Leung KW, Cheng YK, Mak NK, Chan KK, Fan TP, Wong RN. Signaling pathway of ginsenoside-Rg1 leading to nitric oxide production in endothelial cells. *FEBS Lett* 2006;580:3211–6.
- Cheng Y, Shen LH, Zhang JT. Anti-amnesic and anti-aging effects of ginsenoside Rg1 and Rb1 and its mechanism of action. *Acta Pharmacol Sin* 2005;26:143–9.
- Lee WM, Kim SD, Park MH, Cho JY, Park HJ, Seo GS, Rhee MH. Inhibitory mechanisms of dihydroginsenoside Rg3 in platelet aggregation: critical roles of ERK2 and cAMP. *J Pharm Pharmacol* 2008;60:1531–6.
- Liu R, Xing D, Lu H, Wu H, Du L. Pharmacokinetics of puerarin and ginsenoside Rg1 of CBN injection and the relation with platelet aggregation in rats. *Am J Chin Med* 2006;34:1037–45.
- Oh WJ, Endale M, Park SC, Cho JY, Rhee MH. Dual Roles of quercetin in platelets: phosphoinositide-3-kinase and MAP kinases inhibition, and cAMP-dependent vasodilator-stimulated phosphoprotein stimulation. *Evid Based Complement Alternat Med* 2012;2012:485262.
- Roger S, Pawlowski M, Habib A, Jandrot-Perrus M, Rosa JP, Bryckaert M. Costimulation of the Gi-coupled ADP receptor and the Gq-coupled TXA2 receptor is required for ERK2 activation in collagen-induced platelet aggregation. *FEBS Lett* 2004;556:227–35.
- Cimmino G, Golino P. Platelet biology and receptor pathways. *J Cardiovasc Transl Res* 2013;6:299–309.
- Clemetson KJ. Platelets and primary haemostasis. *Thromb Res* 2012;129:220–4.
- Lien LM, Chen ZC, Chung CL, Yen TL, Chiu HC, Chou DS, Huang SY, Sheu JR, Lu WJ, Lin KH. Multidrug resistance protein 4 (MRP4/ABCC4) regulates thrombus formation in vitro and in vivo. *Eur J Pharmacol* 2014;737:159–67.
- Lee JJ, Han JH, Jung SH, Lee SG, Kim IS, Cuong NM, Huong TT, Khanh PN, Kim YH, Yun YP, et al. Antiplatelet action of indirubin-3'-monoxime through suppression of glycoprotein VI-mediated signal transduction: a possible role for ERK signaling in platelets. *Vascul Pharmacol* 2014;63:182–92.
- Lu Y, Li Q, Liu YY, Sun K, Fan JY, Wang CS, Han JY. Inhibitory effect of caffeic acid on ADP-induced thrombus formation and platelet activation involves mitogen-activated protein kinases. *Sci Rep* 2015;5:13824.
- Keane C, Petersen HJ, Tilley DO, Haworth J, Cox D, Jenkinson HF, Kerrigan SW. Multiple sites on *Streptococcus gordonii* surface protein PadA bind to platelet GPIIb/IIIa. *Thromb Haemost* 2013;110:1278–87.
- van der Meijden PE, Feijge MA, Swieringa F, Gilio K, Nergiz-Unal R, Hamulyak K, Heemskerk JW. Key role of integrin alpha(IIb)beta(3) signaling to Syk kinase in tissue factor-induced thrombin generation. *Cell Mol Life Sci* 2012;69:3481–92.
- Leclerc JR. Platelet glycoprotein IIb/IIIa antagonists: lessons learned from clinical trials and future directions. *Crit Care Med* 2002;30:S332–40.
- Payrastre B, Missy K, Trumel C, Bodin S, Plantavid M, Chap H. The integrin alpha IIb/beta 3 in human platelet signal transduction. *Biochem Pharmacol* 2000;60:1069–74.